AIP | Advances

Using magnetic nanoparticles to probe protein damage in ferritin caused by freeze concentration

E. F. Chagas, S. Correia Carreira, and W. Schwarzacher

Citation: AIP Advances **5**, 117201 (2015); doi: 10.1063/1.4935261
View online: http://dx.doi.org/10.1063/1.4935261
View Table of Contents: http://scitation.aip.org/content/aip/journal/adva/5/11?ver=pdfcov
Published by the AIP Publishing

Using magnetic nanoparticles to probe protein damage in ferritin caused by freeze concentration

E. F. Chagas,[1,2,a] S. Correia Carreira,[2] and W. Schwarzacher[2]
[1]*Instituto de Física, Universidade Federal de Mato Grosso, 78060-900, Cuiabá-MT, Brazil*
[2]*H. H. Wills Physics Laboratory, University of Bristol, Bristol, United Kingdom*

(Received 4 June 2015; accepted 22 October 2015; published online 2 November 2015)

We demonstrate a method for monitoring the damage caused to a protein during freeze-thawing in the presence of glycerol, a cryo-protectant. For this work we synthesized magnetite nanoparticles doped with 2.5% cobalt inside the protein ferritin (CMF), dissolved them in different concentration glycerol solutions and measured their magnetization after freezing in a high applied field (5 T). As the temperature was raised, a step-like decrease in the sample magnetization was observed, corresponding to the onset of Brownian relaxation as the viscosity of the freeze-concentrated glycerol solution decreased. The position of the step reveals changes to the protein hydrodynamic radius that we attribute to protein unfolding, while its height depends on how much protein is trapped by ice during freeze concentration. Changes to the protein hydrodynamic radius are confirmed by dynamic light scattering (DLS) measurements, but unlike DLS, the magnetic measurements can provide hydrodynamic data while the solution remains mainly frozen. [http://dx.doi.org/10.1063/1.4935261]

The study of biomolecules during freezing is an interesting branch of biophysics that is fundamental to food preservation[1] and plays an important role in pharmaceutical research, where freezing reduces the risk of microbial contamination[2] and simplifies transportation and storage.[2,3] Despite this subject's importance there are many open questions, mainly due to the difficulty of obtaining information from the sample in the frozen state.[4,5] Here we demonstrate a method of studying protein damage in freeze-concentrated solutions while frozen. We report a study of damage to the protein ferritin during freeze-thawing cycles in glycerol-water mixtures. The proteins contain cobalt-doped magnetite nanoparticles to facilitate magnetic monitoring of their orientation. Our method is based on an approach used previously to probe protein Brownian rotation close to the glass transition of a freeze-concentrated buffer.[6]

The protein ferritin is assembled from 24 subunits that form a spherical nano-cage with internal and external diameters ~ 8 nm and ~ 12.5 nm respectively.[7–9] Native ferritin has a ferrihydrite core, but this may be replaced by the synthesis of a variety of materials,[10] including magnetite[11] and magnetite doped with cobalt nanoparticles,[12] as used in this work. Ferritin is very stable and can withstand a wide range of pH (4-12) and relatively high temperatures.[8]

Ferritin incorporating magnetite nanoparticles doped with 2.5% Co (CMF) was prepared using a published procedure.[12] The preparation of water/glycerol/CMF solutions is described in the supplemental material.[13] We designate samples by their glycerol concentration followed by the concentration of CMF in μM. For example 1M-0.9 is a sample containing 1M glycerol and 0.9 μM CMF. Field-cooled (FC) and zero field-cooled (ZFC) magnetic susceptibility measurements (not shown) of sample 1M-0.9 between 10 and 250K indicated a blocking temperature (T_B) of ~ 67K for our CMF in a good agreement with previous work.[12] Magnetic measurements were performed using a SQUID magnetometer (MPMS-XL Quantum Design, San Diego, CA).

[a]Email address: efchagas@fisica.ufmt.br

To study its rotational dynamics, each CMF sample was freeze-concentrated and field-cooled in a high magnetic field (5 T) to 170 K.[13] During freeze concentration, water is removed from the glycerol solution in the form of ice. CMF segregates to the remaining glycerol solution. During field-cooling, the 5 T applied field physically rotates the CMF to (partially) align the easy axis of each nanoparticle with the external field. The viscosity of the freeze-concentrated glycerol increases as the temperature is lowered, and at 170 K the CMF alignment is effectively fixed.

M was then measured in 0.05 T applied field as the temperature was raised. Raising the temperature decreases the viscosity of the freeze-concentrated glycerol solution again. This allows M to relax by Brownian rotation, since the 0.05 T applied field is insufficient to maintain the initial alignment of the CMF easy axis with the field. The Brownian rotation gives rise to a step-like decrease in M, as seen clearly in Figure 1. T_R corresponds to the start of the step and is therefore the temperature at which the timescale for Brownian rotation reaches τ_M, the timescale of the magnetic measurements ($\sim$ 100 s). Note that at T_R the timescale for Néel relaxation is typically only of the order of 1-10 μs, i.e. our sample is truly superparamagnetic.

Figure 2(a) shows T_R as a function of the ratio of the initial CMF to glycerol concentration ratio. As this ratio increases, T_R increases. We attribute the increase in T_R to an increase in a, the protein hydrodynamic radius. Our reasoning is as follows: the timescale for Brownian rotation is proportional to ζ/k_BT, where ζ is the rotational friction coefficient.[6] At T_R, this becomes $\tau_M \propto \zeta/k_BT_R$, which means $T_R \propto \zeta$. Assuming that the Stokes equation applies, $\zeta = 8\pi\eta a^3$ where η is the (T-dependent) viscosity of the freeze-concentrated glycerol solution. Hence $T_R \propto \eta a^3$. Since η decreases with increasing T, an increase in T_R imples an increase in a. Note that we have not included a dependence of η on glycerol concentration in this argument because we expect the glycerol concentration after freeze concentration to be determined only by the (supplemented) phase diagram[14,15] for glycerol-water mixes and therefore to be independent of the initial glycerol concentration. Otherwise, η would be higher for a higher initial glycerol concentration, which would give the opposite trend in T_R to that observed. We therefore still need to assume that a increases as the initial CMF to glycerol concentration ratio increases, in order to explain our results.

We further considered an alternative to an increase in a as an explanation for the increase in ζ for samples with a higher CMF to glycerol concentration ratio, namely that the increase in ζ is caused by finite size effects. Increasing the CMF to glycerol ratio would decrease the volume of freeze concentrated solution available to surround each protein, and if the thickness of the fluid shell became significantly less than a, this would also lead to an increase in ζ. Although we cannot completely rule out a contribution from this effect, calculations showed that it could not explain our results.[13]

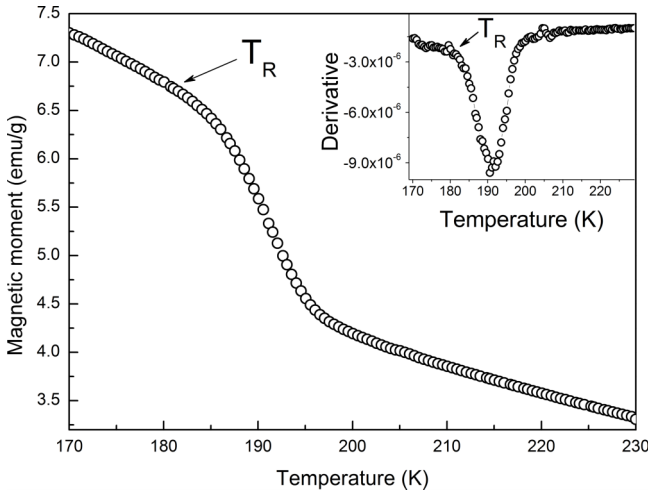

FIG. 1. –FC magnetic susceptibility measurements for sample 1M-0.9, normalized by the mass of CMF in the sample. The FC curve shows the step-like decrease in M due to Brownian rotation. The inset shows the derivative of the FC curve, which is used to estimate T_R.

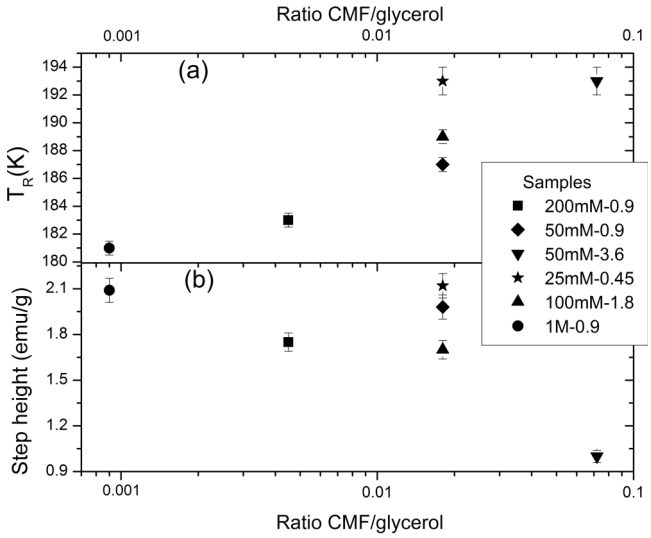

FIG. 2. (a) T_R and (b) step height (see Figure 1) as a function of the initial CMF to glycerol ratio for freeze-concentrated and field-cooled samples, plotted logarithmically because of the range of values involved. Samples are labelled by their glycerol concentration followed by the concentration of CMF in μM. For example 1M-0.9 is a sample containing 1M glycerol and 0.9 μM CMF. Errors were calculated on the basis of estimates of the precision with which T_R, step height and the relevant masses/concentrations could be determined.

During the freeze-concentration procedure, CMF could get trapped in ice or irreversibly adsorbed to it. In either case the quantity of CMF that is free to rotate as a particular sample is warmed after field-cooling will decrease. This will result in a decrease in the height of the step observed at T_R. Figure 2(b) shows the height of this step, as a function of the CMF to glycerol concentration ratio. The step is calculated as the difference between the FC and ZFC magnetizations measured at 180K, normalized to the mass of CMF in the sample. From Figure 2(b), there is some evidence that the amount of CMF trapped during the freeze concentration process, like the hydrodynamic radius a, increases with increasing ratio of CMF to glycerol. When this ratio is increased even further (sample 5mM-0.9 containing 5 mM glycerol and 0.9 μM CMF), no step is observed.

The most likely reason for the observed increase in a with increasing CMF to glycerol ratio would be partial denaturing of the protein. The 24 sub-units making up the ferritin cage each

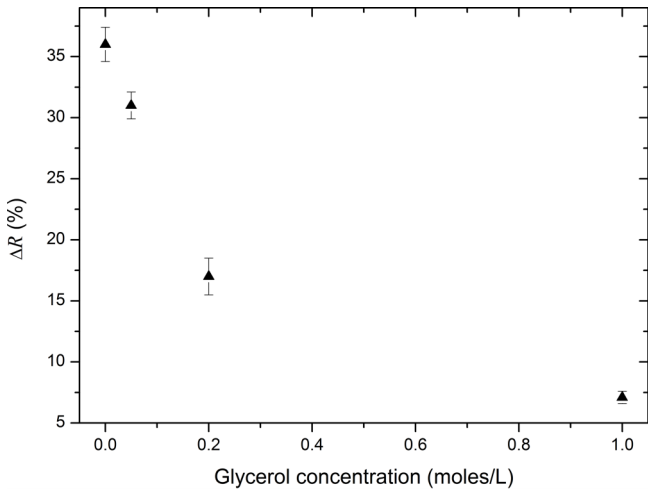

FIG. 3. Percentage increase in protein hydrodynamic ratio a following plunge-freezing to 120K and subsequent thawing as a function of the initial glycerol concentration. The CMF concentration in each case was 0.9 μM.

TABLE I. T_R and step height data for selected samples showing the changes following storage, thawing and renewed freeze-concentration.

	T_R(K)			Step height (emu/g)		
Sample	Initial measurement	After storage	After additional thawing and re-freezing	Initial measurement	After storage	After additional thawing and re-freezing
1M-0.9	181±0.5	180±0.5	183±1.5	2.09±0.08	1.5±0.08	1.6±0.08
200mM-0.9	183±0.5	185±0.5	186±0.5	1.75±0.06	1.3±0.06	1.1±0.06
50mM-0.9	187±0.5	188±0.5	196±0.5	1.98±0.08	1.6±0.08	0.72±0.08

consist of five helices,[16] and the freeze concentration process could cause their partial unfolding. To confirm an increase in a by a different method, we also carried out dynamic light scattering (DLS) measurements to determine the percentage change in the diffusion coefficient D_t of CMF in water and different concentration glycerol solutions before and after plunge freezing in liquid nitrogen and subsequent thawing. In each case, the freeze-thawing process led to a significant decrease in D_t, implying a corresponding increase in a, as shown in Figure 3. Furthermore, the increase was greater the higher the CMF to glycerol ratio, in complete agreement with our magnetic studies.

Although both DLS and our magnetic method can detect increases in a, the advantage of the latter is that it does not require the solution to be thawed to make the measurement. We took advantage of this property to study freeze-concentrated CMF solutions stored at low temperature. Samples 1M-0.9, 200mM-0.9, and 50mM-0.9 were measured again after storage for two weeks in a frozen state at 253K, then warmed to room temperature for 5 min, before being freeze-concentrated a second time, and field-cooled. The results are shown in Table I (the initial measurement data are a subset of those shown in Figure 2.).

In each case T_R after storage was, within error, the same as T_R before, suggesting that there was no additional damage during storage to the protein that remained in solution. However, the step height after storage was significantly lower than the step height before, suggesting that during storage protein adsorbs to ice. After warming to room temperature and repeating the freeze-concentration procedure, however, T_R increased corresponding to a further increase in a, presumably caused by additional protein denaturation. Furthermore, in two out of the three samples, thawing and re-freezing also led the step height to decrease significantly, suggesting that denaturation makes the CMF more likely to be trapped in or adsorb to ice. The decrease is greater for sample 50mM-0.9 where the volume of ice in the freeze-concentrated solution is greater, than for sample 200 mM-0.9. Interestingly, for sample 1M-0.9 the step height actually increases following thawing and re-freezing, which we attribute to the release of CMF that was adsorbed to ice rather than segregated to the freeze-concentrated glycerol solution at the end of the storage stage. This increase clearly outweighs any increased tendency for the partially denatured CMF to be trapped in or adsorb to the ice formed when this sample is freeze-concentrated a second time.

CONCLUSION

We demonstrate that it is possible to obtain information about damage caused to a protein by the freeze-concentration process via magnetic measurements. The main advantage of this method over e.g. DLS is that it can detect damage to a protein while the solution remains partially frozen. This means that it could be used to evaluate damage caused by storage at different temperatures and for different times, or to monitor the damage caused at different stages of the freeze-thawing process.

ACKNOWLEDGMENTS

This work was supported by CAPES (Brazilian funding agency).

[1] M. Karel and D. B. Lund, *Physical Principles of Food Preservation* (Marcel Dekker, Inc., 2003), p. 276.
[2] S. K. Singh, P. Kolhe, A.P. Mehta, S. C. Chico, A. L. Lary, and M. Huang, Pharm. Res. **28**, 873 (2011).
[3] B. S. Bhatnagar, R. H. Bogner, and M. J. Pikal, Pharm. Dev. and Technol. **12**, 505 (2007).
[4] D. A. Hall, D. C. Maus, G. J. Gerfen, S. J. Inati, L. R. Becerra, F. W. Dahlquist, and R. G. Griffin, Science **276**, 930 (1997).
[5] G. B. Strambini and M. Gonnelli, Biophys. J. **92**, 2131 (2007).
[6] J.-C. Eloi, M. Okuda, S. E. Ward Jones, and W. Schwarzacher, Biophys. J. **104**, 2681 (2013).
[7] R. J. Hoare, P. M. Harrison, and T. G. Hoy, Nature **255**, 653 (1975).
[8] J. S. Colton, S. D. Erickson, T. J. Smith, and R. K. Watt, Nanotechnology **25**, 135703 (2014).
[9] L. Schoonen and J. C. M. van Hest, Nanoscale **6**, 7124 (2014).
[10] I. Yamashita, K. Iwahori, and S. Kumagai, Biochim. Biophys. Acta **1800**, 846 (2010).
[11] F. C. Meldrum, B. R. Heywood, and S. Mann, Science **257**, 522 (1992).
[12] M. Okuda, J.-C. Eloi, A. Sarua, S. E. Ward Jones, and W. Schwarzacher, J. Appl. Phys. **111**, 07B519 (2012).
[13] See supplementary material at http://dx.doi.org/10.1063/1.4935261 for details of sample preparation, freeze concentration, FC and ZFC measurement procedures, additional calculations and DLS measurements.
[14] S. Ablett, M. J. Izzard, and P. J. Lillford, "Differential scanning calorimetric study of frozen sucrose and glycerol solutions," J. Chem. Soc. Faraday Trans. **88**, 789 (1992).
[15] K. Murata and H. Tanaka, Nature Mater. **11**, 436 (2012).
[16] Y. Zhang and B. P. Orner, Int. J. Mol. Sci. **12**, 5406 (2011).